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Measuring Nitrate in Plant Cells by *in Vivo* NMR Using Gd^{3+} as a Shift Reagent

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NMR investigations of nitrate in plant cells and tissues have hitherto been limited by the indistinguishability of the signals from intracellular and extracellular nitrate. Gd^{3+} is shown to be an effective shift reagent for ^{14}N and ^{15}N nitrate NMR signals, resolving the internal and external nitrate signals in plant tissues, including cell suspensions and root material. However, time-course experiments show that, while the use of Gd^{3+} allows nitrate levels to be monitored over extended periods, it also has adverse effects on growth and nitrate uptake. Accordingly, a number of chelated forms of gadolinium were investigated, and it is concluded that the NMR contrast agent Gd(DTPA-BMA) is likely to be a suitable shift reagent for physiologically relevant studies of nitrate transport in roots. © 1996 Academic Press, Inc.

INTRODUCTION

The chemical shifts of the NMR signals from many of the physiologically interesting anions and cations are only weakly dependent on their environment. As a result, the contributions from the intracellular and extracellular pools of such ions often overlap in the spectra of cells and tissues, complicating the interpretation of ion transport experiments. This problem was solved for several physiologically important cations, including K^+, Na^+, and NH_4^+, by the introduction of paramagnetic shift reagents (*1*), and the transport of these and other cations has now been studied in a wide range of living systems by *in vivo* NMR (*2*).

While some of the physiologically important anions that are detectable by NMR, notably bicarbonate and phosphate, have pH-dependent shifts that can be exploited in ion transport studies, others do not, and there has been relatively little interest in addressing this problem by the development of appropriate shift reagents. Chloride and nitrate are the two anions for which this approach would be most useful, and it was recently shown that the Co(II) ion could be used to separate intracellular and extracellular chloride signals (*3*). In fact, severe quadrupolar broadening restricts the application of *in vivo* ^{35}Cl NMR to the detection of chloride in compartments, such as plant cell vacuoles, with low protein content. In contrast, the ^{14}N and ^{15}N NMR signals from tissue nitrate are fully detectable, and a shift reagent for nitrate would be expected to facilitate nitrate transport studies in microorganisms and plant tissues.

Nitrate is an important source of nitrogen for many plants, and nitrate uptake and storage are important phenomena (*4*). The main techniques currently available used for studying these processes are ^{13}N and ^{15}N tracer methods (*5, 6*) (which require compartmental analysis and fitting of fluxes) and nitrate-specific electrodes for monitoring levels in single cells (*7, 8*) (which is restricted to cells accessible from the surface). *In vivo* NMR methods have also been used to study nitrate in plants (*9, 10*) and have advantages of being direct, continuous, and noninvasive. To date, these studies have been limited by the need to use nitrate-free solutions because of the superposition of the intracellular and extracellular signals when nitrate is present in the medium. It should, however, be possible to eliminate this problem by developing appropriate shift reagents, and here we report that this can be achieved using gadolinium or gadolinium chelates.

MATERIALS AND METHODS

Plant Material

Carrot (*Daucus carota,* L.) cells were grown as described previously (*11*), and soybean (*Glycine max,* L.) cells were the gift of Dr D. E. Hanke (Department of Plant Sciences, Cambridge University) (*12*). All cultured cells were harvested by filtration as described (*11*). Maize (*Zea mays,* L. cultivars W 7551 and LG11) seeds were germinated for 48–60 h at 25–30°C on absorbent paper soaked in 0.1 m*M* $CaSO_4$. Nitrate-uptake mechanisms were induced by exposure of the seedlings to 10 m*M* KNO_3/0.1 m*M* $CaSO_4$ for a further 12–18 h. For uptake experiments, 5 mm root tips or 2.5 cm long root segments taken from 5 mm behind

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the tip were cut and kept in oxygenated buffer solutions containing 10 m*M* MES/0.1 m*M* $CaSO_4$ at pH 6.0.

NMR Spectroscopy

^{14}N, ^{15}N, and ^{31}P NMR spectra were recorded at 7.05 T on a Bruker CXP 300 spectrometer and at 9.4 T on a JEOL GX-400 spectrometer. Spectra from cell suspensions were recorded with 20 mm probeheads and spectra from root tissues were recorded with 10 mm probeheads. ^{14}N spectra were acquired using 90° pulses and recycle times greater than 200 ms. Cell suspensions were maintained in a well-oxygenated state using an airlift system (*13*); excised root tissues were oxygenated either by airlift or by continuous perfusion.

Nitrate shifts in simple solutions were characterized using coaxial NMR tubes (Wilmad Glass Company); paramagnetic species were always added to the solution in the outer of the two tubes. The gadolinium concentration, the nitrate concentration, and the total ionic strength were varied independently in each of three titrations using KNO_3, $GdCl_3$, $Gd(NO_3)_3$, and KCl. Bulk susceptibility shifts were measured by recording the ^1H spectrum of the coaxial sample, and the shift measured in the water resonance in parts per million was subtracted from the shift seen in the nitrogen spectrum of the same sample. This correction does not take into account any specific shift in the ^1H resonance of water induced by the Gd^{3+} but this specific shift is known to be small [0.003 ppm/m*M* Gd^{3+} (*14*)] at the low Gd^{3+} concentrations used here.

Reagents

Analytical-grade gadolinium (III) salts were obtained from Aldrich Chemical Company and "omniscan" (the bis-*N*-methylamide of Gd.diethylene triaminepentaacetate, Gd.DTPA-BMA) was obtained from Nycomed (Oslo). Derivatives of dipicolinic acid were synthesized according to published protocols (*15, 16*).

RESULTS AND DISCUSSION

By analogy with the work on chloride [(*3*) and references therein], we considered the use of several paramagnetic transition metal and lanthanide cations as potential shift reagents for nitrate. Previous work provides some relevant data including chemical shifts induced in the nitrate resonance by paramagnetic cations in nonaqueous solutions (*17*) and equilibrium constants for nitrate binding to the rare earth cations in water (*18*). We found Gd^{3+} to be the most effective of those tried (Mn^{2+}, $Fe^{2}+$, $Co^{2}+$, Cu^{+2}, Dy^{3+}, Pm^{3+}, Gd^{3+}, Sm^{3+}) since low concentrations of Gd^{3+} induced substantial shifts to high frequency with only modest broadening of the nitrate signal (Fig. 1). Spectra of ^{15}N-labeled nitrate taken under the same conditions show the same induced shift in parts per million as that in ^{14}N spectra. Lorentzian lineshapes are observed here (and in all spectra in this study) for ^{14}N and ^{15}N signals of nitrate in the presence of Gd^{3+}. The integrated areas of spectra taken before and after adding Gd^{3+} are the same so that the addition of Gd^{3+} does not affect the NMR visibility of nitrate in solution. This was observed for analytical samples such as that in Fig. 1, as well as for the cell suspensions and root tissues described below. In contrast to the shifts induced by the paramagnetic reagents used for *in vivo* spectroscopic studies of cations [reviewed in (*1, 2*)], the symmetry of the half-filled *f* orbitals of Gd^{3+} indicates that the shift induced in nitrate signals is a contact shift, and, therefore, analogous to the shifts induced by Gd^{3+} in the ^1H and ^{17}O spectra of water (*14*).

We characterized the shift induced in the nitrate resonance by Gd^{3+} in aqueous solutions as a function of the concentration of gadolinium and ionic strength. Figure 2a shows the shift induced in the ^{14}N nitrate signal as a function of Gd^{3+} concentration with the total ionic strength and the nitrate concentration kept constant. The shifts were referenced to the unshifted peak and were corrected for bulk-susceptibility effects by using the ^1H spectrum of the same sample (see Materials and Methods). The susceptibility shifts for Gd^{3+} and for the complexes studied below were approximately 0.1 ppm/m*M* Gd^{3+} and were in the same direction as the contact shift (i.e., enhanced resolution). The observation of linearity in Fig. 2a implies that the fraction bound is proportional to the activity of Gd^{3+} throughout this concentration range, since the induced shift is proportional to the fraction of nitrate bound to Gd^{3+} for a rapidly exchanging system (*19*). The induced shift was also found to be independent of the nitrate concentration between 1 and 100 m*M* at a constant Gd^{3+} concentration (10 m*M*) and constant ionic strength ($I = 0.16$), showing that the binding constant was low. These data are in agreement with previous work (*18*) in which the complexation of trivalent lanthanide ions by nitrate in aqueous solutions was found to be of low affinity.

In contrast to the result in Fig. 2a, the shift induced in the nitrate resonance increased nonlinearly with increasing concentrations of Gd^{3+} when ionic strength was not held constant. The dependence of the gadolinium-induced shift on ionic strength is expected to vary as

$$\log_{10}\Delta\omega = \log_{10}(K[Gd^{3+}]\Delta\omega_b) - 1.018z\{\sqrt{(I)}/[1 + \sqrt{(I)}]\}, \quad [1]$$

where $\Delta\omega$ is the shift induced by gadolinium, $\Delta\omega_b$ is the shift of gadolinium-bound nitrate, K is the binding constant,

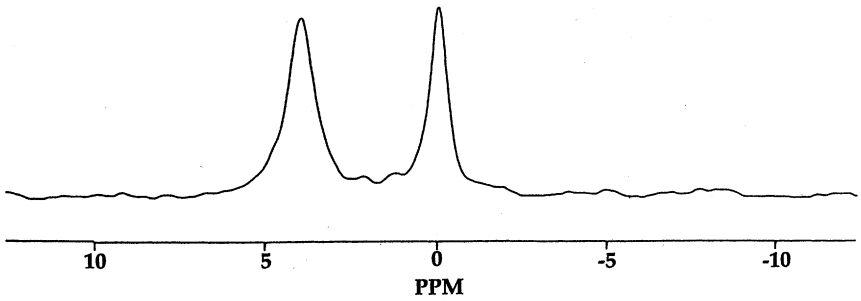

FIG. 1. The effect of gadolinium on the chemical shift of the ^{14}N NMR signal from nitrate. This ^{14}N spectrum was recorded from 15 m*M* $K^{14}NO_3$/ 15 m*M* $K^{15}NO_3$/5 m*M* $GdCl_3$ in the presence of a coaxial capillary containing 1 *M* $NaNO_3$. Gadolinium caused a 4 ppm shift to high frequency in the nitrate signal relative to the signal from the capillary of which approximately 10% is due to bulk susceptibility.

and z is the effective charge on the lanthanide ion. This expression is obtained from the extended Debye–Hückel equation describing the effect of ionic strength (I) on the interaction of charged species in solution (*18*), assuming that the induced shift is proportional to the fraction of nitrate bound to Gd^{3+} as in a rapidly exchanging system (*19*). Figure 2b shows the observed dependence of the gadolinium-induced shift on ionic strength. The first data points fall on a straight line with negative slope, as expected from Eq. [1], and the data at higher ionic strength deviate progressively from linearity (as the Debye–Hückel approximations become inapplicable). These results together are consistent with an inner sphere interaction of the form

$$Gd^{3+} + NO_3^- \rightleftharpoons (Gd \cdot NO_3)^{2+},$$

with low binding constant, undergoing rapid exchange. It is also clear that ionic strength needs to be controlled if the induced shifts are to be properly characterized in this and other systems where a charged shift reagent interacts with another charged species.

The practicality of using gadolinium *in vivo* was first tested using suspension cultures of carrot and soybean. Figure 3 shows the ^{14}N spectrum of a carrot-cell suspension in the presence of 20 m*M* Gd^{3+}, and it can be seen that the intracellular nitrate signal was resolved from the external signal. The NMR visibility of nitrate has been previously determined by Belton *et al.* (*9*), who showed that the ^{14}N NMR signals from intracellular nitrate in root samples correspond fully to the total concentration of nitrate. Therefore, this approach makes possible the direct measurement of intracellular nitrate levels in the presence of external nitrate. However, in order to induce sufficient shift to resolve external from external signals in these cell suspensions, it was

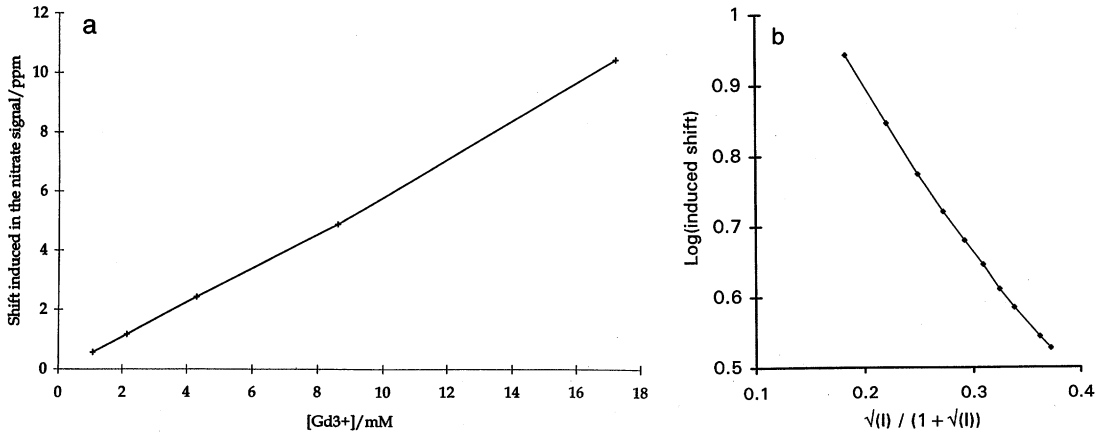

FIG. 2. The effect of (a) the concentration of gadolinium and (b) ionic strength on the shift induced by Gd^{3+} in the ^{14}N NMR nitrate signal. The dependence on the gadolinium concentration was measured using 30 m*M* KNO_3 at a constant ionic strength of 0.13 *M*, while the effect of the ionic strength was measured in solutions containing 8.33 m*M* $Gd(NO_3)_3$ and concentrations of KCl in the range 0–300 m*M*. The shifts were measured using a coaxial sample with the solution containing gadolinium in the outer compartment, and they were corrected for the bulk-magnetic-susceptibility effect using the ^1H NMR signals from the water in the aqueous solutions.

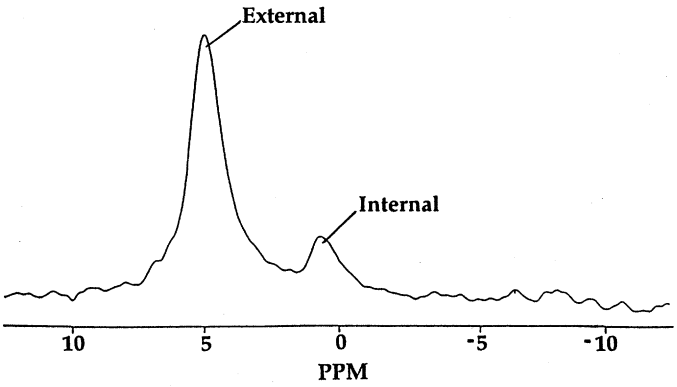

FIG. 3. ^{14}N NMR spectrum of a carrot-cell suspension in the presence of gadolinium. The cells were suspended in 10 m*M* MES/25 m*M* $NaNO_3$/0.1 m*M* $CaSO_4$, pH 6.0, and the shift was induced with 20 m*M* $GdCl_3$. Zero parts per million on the chemical-shift scale corresponds to the position of the combined intracellular and extracellular signals in the absence of gadolinium.

necessary to add substantially higher levels of Gd^{3+} than needed in simple solutions or in maize-root tissue samples (see below). Acidification of the external medium increased the observed shift, and, since the gadolinium-induced shift in simple solutions is independent of pH between 3.0 and 6.0, we concluded that the effectiveness of the shift reagent was reduced by binding to cell walls. In fact, the cell walls of dicotyledonous species contain negatively charged moieties (uronic acids) to which Gd^{3+} would be expected to bind strongly, at neutral but not acidic pH values, thus lowering its concentration in solution.

We next used maize-root tips and maize-root segments for a series of time-course experiments to test whether nitrate uptake could be monitored *in vivo* in the presence of gadolinium. Figure 4 shows representative ^{14}N NMR spectra from such an experiment on root tips. Each spectrum in Fig. 4 was acquired in 20 min., although spectra with sufficient signal-to-noise to quantify internal nitrate levels were obtainable in as little as 5 min. In contrast to the results obtained with cell suspensions (Fig. 3), adequate resolution of the intracellular and extracellular nitrate signals was obtained with a gadolinium concentration of only a few millimolar. Moreover, the chemical shifts and linewidths of the signals were constant throughout extended time courses, suggesting minimal uptake of Gd^{3+} by the tissue.

^{14}N spectra from samples of root segments from three-day-old maize seedlings, grown and maintained during NMR measurements under the same conditions as those for the root tips, gave spectra in which internal and external nitrate signals were also fully resolved. Maize-root segments showed higher internal nitrate signals consistent with previous work (*9*). Since the intracellular volume in such samples is comparable to the total extracellular volume, the intracellular nitrate concentration is easily estimated directly from the ratio of the intensities of the internal and external nitrate signals. Thus, this approach can be used to follow nitrate levels and net transport in living tissue. Analogous experiments performed with [^{15}N] nitrate show that this approach can be used with both isotopes, although the time resolution is less good because of the slower spin–lattice relaxation of ^{15}N nitrate.

While the data in Figs. 1–4 show that gadolinium is an effective shift reagent for nitrate, it is important for physiological studies of nitrate uptake in plants that it should not interfere with transport processes. In an earlier ^{31}P NMR study (*20*), it was shown that exposure to millimolar concentrations of gadolinium caused some uptake of the lanthanide ion but that this had no effect on the cellular metabolism and energetics. In agreement with these findings, ^{31}P spectra of three-day-old maize-root tips taken before and after exposure to 5 m*M* Gd^{3+} for 4 h showed that levels of nucleotide triphosphates were unaffected by exposure to Gd^{3+} and that cytoplasmic and vacuolar pH values were likewise unperturbed (data not shown).

In order to assess the effect of Gd^{3+} on the uptake of nitrate, root tips were excised from three-day-old maize seedlings and maintained in oxygenated buffer solutions at pH 6 in the presence of 0, 0.5, or 5 m*M* $GdCl_3$. Nitrate uptake under these three conditions was compared by periodically removing the root tips from the incubation solutions, placing them in nitrate-free buffer solution in an NMR tube

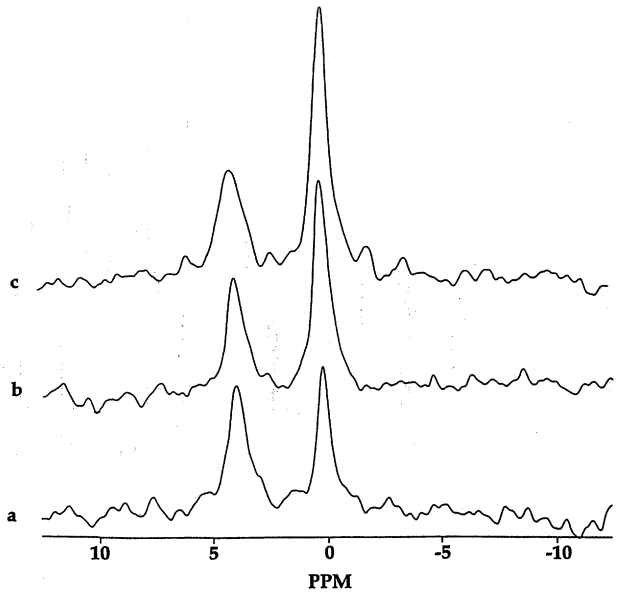

FIG. 4. Nitrate uptake by maize-root tips in the presence of gadolinium. The tissue was bathed in an oxygenated medium containing 10 m*M* MES/1 m*M* KCl/0.5 m*M* $CaSO_4$, pH 6.0 in the NMR tube, and spectra were recorded after (a) 3.5 h, (b) 6 h, and (c) 13 h exposure to 5 m*M* $NaNO_3$/5 m*M* $GdCl_3$.

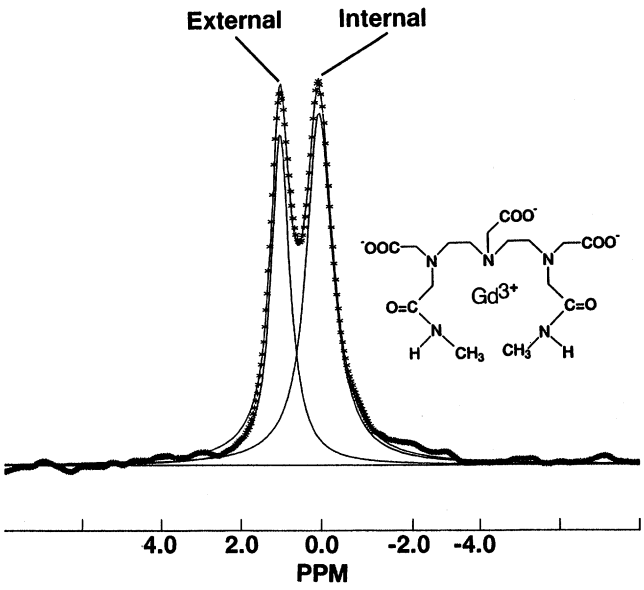

FIG. 5. ^{14}N NMR spectrum (experimental data points, ×) of maize-root segments in the presence of the gadolinium chelate Omniscan (structure inset). Also, the results of fitting the spectrum to two Lorentzian lines (solid lines). The tissue was bathed in an oxygenated medium containing 10 m*M* MES/10 m*M* KNO_3/0.1 m*M* $CaSO_4$, pH 6.0, and the shift was induced by 10 m*M* Gd(DTPA-BMA).

and recording ^{14}N spectra. Exposure to 5 m*M* $GdCl_3$ inhibited nitrate uptake substantially, and even 0.5 m*M* $GdCl_3$ caused significant inhibition. This inhibition was seen within 1 h and continued throughout the time course. In fact, trivalent lanthanide cations including Gd^{3+} are known to inhibit calcium movement across the plasma membrane of maize roots (*21*), and this suggests that raising the external calcium concentration could counteract the inhibition of nitrate uptake. However, the uptake of nitrate by maize-root tips incubated in solutions containing 0.5 m*M* $GdCl_3$, 10 m*M* KNO_3, and 5 m*M* MES at pH 6 was unaffected by increasing the concentration of calcium from 0.1 to 10 m*M* $CaCl_2$ (data not shown). Since changes in energization, pH gradients, and calcium uptake are not apparently the cause of the observed inhibition of nitrate uptake, we postulate that it is related to the observation that trivalent cations inhibit root growth (*22*). We conclude that Gd^{3+} itself, despite its favorable NMR properties, is unsuitable for physiological studies and that chelated forms should be investigated.

A priori considerations suggest: (i) that a gadolinium chelate capable of inducing a shift in the nitrate signal would either be neutral or positively charged; and (ii) that its charge would be less than +3, in view of the interesting observation of Kinraide and co-workers (*22*), who found that root growth in wheat was inhibited by a trivalent complexed cation, $[Co(ethylene\text{-}diamine)_3]^{3+}$, as well as by simple trivalent cations such as Al^{3+} and Gd^{3+}. Thus, candidate shift reagents for nitrate include: (i) several complexes that were developed as contrast agents for NMR imaging, e.g., Gd(III) DTPA–BMA or "Omniscan" (*23, 24*); (ii) soluble texaphyrin complexes with a defined nitrate binding site and a net charge +2 (*25*); and (iii) simple chemical complexes, such as those with dipicolinic acid, which is known to bind tightly to lanthanides (*26*).

Terbium(III), and by strong implication gadolinium, binds up to three molecules of dipicolinic acid (pyridine-2,6-dicarboxylic acid). We observed substantial, though decreased, gadolinium-induced contact shifts in the nitrate resonance when 16 to 40 m*M* dipicolinate was added to 16 m*M* Gd^{3+}. Under these conditions, the concentration of free gadolinium is expected to be very low. While this shows that Gd complexes with net charge of less than three can induce shifts in the nitrate signal, we found that precipitation at pH values in the physiologically interesting range makes dipicolinate an unsuitable chelator for biological studies. We attempted to address this problem by synthesizing substituted derivatives of dipicolinic acid with OH, Cl, or NH_2 in position 4 of the pyridine ring (*15, 16*). None of these attempts to increase the solubility were successful, and it was concluded that this was not a promising approach.

However, no such problems were encountered with the DTPA–BMA complex of gadolinium. Figure 5 shows that 10 m*M* Omniscan induced a shift of approximately 1 ppm in the position of the extracellular nitrate signals in a sample of maize-root segments. The spectrum gave a good fit to two Lorentzian lines (*27*), to within the signal-to-noise, and the two components were also easily separable by resolution enhancement. The uptake of nitrate into maize-root segments was followed for 15 h without loss of resolution, and experiments similar to those with Gd^{3+} showed that nitrate uptake and growth were unaffected by the presence of the complex.

In conclusion, the use of Omniscan permits physiologically relevant studies of nitrate transport by *in vivo* NMR, and we expect that the availability of this shift reagent will stimulate more detailed studies of nitrate uptake in plant roots (*9*) than has been possible previously.

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